IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	
Saiko HOSOKAWA et al.	:	Atty Docket 00177/530714
Serial No. 09/467,903	:	Group Art Unit 1644
Filed December 21, 1999	:	Examiner R. Schwadron
HUMAN MONOCLONAL ANTIBODY SPECIFICALLY BINDING TO SURFACE ANTIGEN OF CANCER CELL MEMBRANE	:	

APPELLANTS' BRIEF IN SUPPORT OF APPEAL

Honorable Commissioner of Patents and Trademarks,
Washington, D.C. 20231

ATTENTION: Board of Patent Appeals and Interferences

Sir:

This Brief is in furtherance of the Notice of Appeal, filed in this case on April 10, 2001.

The fees required under 37 C.F.R. § 1.17, and any required Petition for extension of time for filing this Brief and fees therefor, are dealt with in the accompanying PATENT OFFICE FEE TRANSMITTAL FORM.

This Brief is transmitted in triplicate.

I. REAL PARTY IN INTEREST

The real party in interest in this appeal is the party named in the caption of this brief.

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II. RELATED APPEALS AND INTERFERENCES

There are no appeals or interferences that will directly affect, or be directly affected by, or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS.

The status of the claims in this application are:

A. TOTAL NUMBER OF CLAIMS IN THE APPLICATION

Total claims in the application are claims 1-49.

B. STATUS OF ALL THE CLAIMS

1. Claims 1-29 have been canceled without prejudice.
2. Claims 30-49 have been added.
3. No claims have been withdrawn from consideration.
4. Claims 30-49 are pending.
5. No claims are allowed.
6. Claims 30-49 are rejected.

C. CLAIMS ON APPEAL

The claims on appeal are claims 30-49.

IV. THE STATUS OF AMENDMENTS.

There were no amendments filed after the final rejection dated January 10, 2001. Thus, the final status of the claims for appeal is claims 30-49.

VI. SUMMARY OF THE INVENTION.

As disclosed on page 1, lines 4 and 5, of the specification, the present invention relates to an anti-cancer formulation (i.e. pharmaceutical composition and liposome/antibody conjugate). The claimed pharmaceutical composition comprises a therapeutically effective amount of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells and a pharmaceutically acceptable carrier therefor, said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine, said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of the liposome through a maleimide group, said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane, and said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:5 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:6. It is noted that said antibody fragment can alternatively have a variable region of the heavy chain comprising the amino acid sequence shown in SEQ ID No:11 and a variable region of the light chain which comprising the amino acid sequence shown in SEQ ID No:12.

The claimed liposome/antibody conjugate consists essentially of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells, said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine, said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of a liposome through a maleimide group, said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane, and said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:5 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:6. Like the claimed pharmaceutical composition, the antibody fragment of the claimed liposome/antibody conjugate can also alternatively have a variable region of the heavy chain comprising the amino acid sequence shown in SEQ ID No:11 and a variable region of the light chain comprising the amino acid sequence shown in SEQ ID No:12.

Using the claimed pharmaceutical composition or liposome/antibody conjugate noted above, cancerous tissues or organ can be treated continously and over a longer term via "targeting" therapy.

VI. ISSUES ON APPEAL.

The sole issue on appeal is whether claims 30-49 are unpatentable under 35 USC §112, first paragraph, as containing subject matter (specifically "antibody fragments") which was not described in the specification in such a way as to reasonable convey to one skilled in the relevant

art that the inventors, at the time the application was filed, had possession of the claimed invention.

VII. GROUPING OF CLAIMS.

The claims should be separated into four groups, with claims 30-34 and claims 35-39, directed to a pharmaceutical composition, in two separate groups and with claims 40-44 and claims 45-49, directed to a liposome/antibody conjugate, in another two separate groups. Thus, the four groups, directed to (a) claims 30-34, (b) claims 35-39, (c) claims 40-44 and (d) claims 45-49, should be judged independently of one another.

VIII. ARGUMENTS - REJECTION UNDER 35 U.S.C. § 112, FIRST PARAGRAPH.

The sole issue to be resolved with respect to this rejection is whether claims 30-49 contain subject matter, ***specifically antibody fragments such as Fab' or F(ab')$_2$***, which was not described in the specification in such a way as to reasonable convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

Initially, Applicants wish to note to the Appeal Board that in support of the remarks contained hereinbelow, a Declaration from the parent application was submitted under 37 C.F.R. 1.132 and is of record in the application file. Applicants respectfully request the Appeal Board to review the Declaration and references (cited below) in light of the comments set forth herein below.

Applicants believe that the specification clearly teaches one skilled in the art how to use the claimed antibody fragments, such as Fab'. Further, Applicants also strongly believe that it is well known to one skilled in the art how to use other antibody fragments, such as $F(ab')_2$ in view of the state of the art at the time of the priority date of the present application.

Based on the Examainer's comments, the Examiner believes that the use of the term "antibody fragment" encompasses subject matter which includes antibody fragments such as Fv, Fd, or $F(ab')_2$, which are not described in the specification. However, Applicants strongly believe that the specification clearly describes how to use Fab' fragments in the present invention, and how to use $F(ab')_2$ fragments for the purpose of the present invention.

It is well established in patent law that the specification does not need to literally describe "in ipsis verbis" the particular claim language in order for the specification to satisfy the written description requirement of 35 USC § 112, first paragraph. See, for example, *In re Lukach* 169 USPQ 795, 796 (CCPA) 1971. Furthermore, it is sufficient that the specification "convey clearly to one skilled in the art, the information that the Applicant has invented the specific subject matter claimed". See, for example, *In re Wertheim* 191 USPQ 90, 96 (CCPA 1976) and *In re Ruschig* 154 USPQ 118, 123 (CCPA 1967).

It is appropriate for the claims to utilize claim language which does not readily appear in the specification as long as one skilled in the art would ***impliedly*** or ***inherently*** recognize that the Applicant has invented the specific subject matter claimed. In reviewing the teachings of the specification set forth on pages 1, 3, 12, 36, 37 and 41, it is clear that the inventors did

contemplate a pharmaceutical composition or a liposome/antibody conjugate comprising an antibody fragment of the monoclonal antibody.

The specification teaches on page 11, line 18 to page 12, line 17, the method for binding an antibody to the surface of a liposome. It discloses as a preferred method a reaction of a thiolated antibody with a maleimide group existing in a liposome (see page 11, lines 21-24). As methods for thiolation, it teaches (1) the use of SPDP, iminothiolane (methyl-4-mercaptobutyrimidate), or mercaptoalkylimidate, which is conventionally used for thiolation of proteins (page 11, lines 21-24), and (2) reduction of the dithiol group intrinsic to an antibody (page 12, lines 6-9).

The second method (2) mentioned above comprises the use of the thiol group possessed by a Fab' antibody fragment which is formed by the reduction of a $F(ab')_2$ fragment as disclosed on page 12, lines 9-13 and page 36, line 16, page 37, line 5 (Section a. of Example 7) of the specification.

On the other hand, the first method (1) mentioned above, which is addressed to **thiolation of a protein having no thiol group, like $F(ab')_2$** is carried out in a conventional manner as described, for example, by Wright, S. et al., Advanced Drug Delivery Reviews, 3 (1989), pp. 343- 352 (see especially on page 351, lines 18-22 and Table II), and by Traut, R.R. et al., Biochemistry, 12 (1973), p 3266-3273, copies of which have already been made of record in the parent application (Serial No. 08/450,363). Applicants have also demonstrated from the Declaration of T. Tagawa (submitted with the Amendment dated January 26, 1998 in the parent application (Serial No. 08/450,363)) that an Experiment conducted using an antibody-bonded

PEG-modified liposome which was prepared by binding $F(ab')_2$ to a liposome in accordance with the above-mentioned Traut's method is clearly well within the state of the art at the time of the priority date and clearly show that the present inventors contemplated the present invention using other antibody fragments such as Fab' and $F(ab)_2$.

Applicants wish to note to the Board that the antibody fragments of the present invention must contain an antigen binding site for the purpose of the present invention. The current claims recite that the monoclonal antibody fragments must be bound to the surface of a liposome enclosing an anti-cancer agent or toxin and be capable of specifically binding to a surface antigen of a stomach and colon cancer cell membrane. Accordingly, the antibody or fragment thereof used in the claimed invention is defined by specific amino acid sequences.

Applicants also wish to advise the Board that the gist of the present invention resides in the finding of a specific monoclonal antibody defined by the amino acid sequences listed in the Sequence Listing, which can actually bind to the aimed antigen, and not in the finding that antibody fragments can also be used in the same manner as the antibodies themselves for the purpose of the present invention. In other words, it is not necessary under U.S. practice for the present application to teach what is already widely known or recognized by those skilled in the art as of the priority date of the present application. Since it is well known to one skilled in the art that antibody fragments in the field of "targeting therapy" are used in the same manner as the antibody themselves, it clearly shows that the inventors contemplated the use of such fragments, as well as the antibodies themselves in the field of "targeting therapy". In support thereof, Applicants have submitted Dr. Hosokawa's Declaration in which the usefulness of antibody

fragment $F(ab')_2$ in "targeting therapy" is experimentally shown. Further, a copy of the reference, Biochimica et Biophysica Acta (880 (1986) p72-77), which teaches the usefulness of $F(ab')_2$ in "targeting therapy" (see, in particular, Abstract of the article), as well as demonstrates that the use of $F(ab')_2$ in "targeting therapy" was already widely known and recognized by those skilled in the art as of the priority date of the present application. In other words, the enclosed Declaration and reference clearly demonstrate that the use of antibody fragments in "targeting therapy" as contemplated by the inventors was well known to one skilled in the art at the time of the invention.

Thus, since it is clear that the present inventors contemplated the use of all antibody fragments capable of specifically binding to the surface antigen of stomach and colon cancer cells for "targeting therapy", Applicants respectfully submit that the rejection of claims 30-49 under 35 USC § 112, first paragraph, cannot be sustained and should be withdrawn.

For the foregoing reasons, it is strongly urged that the final rejection is untenable and should be reversed.

IX. APPENDIX OF CLAIMS

The text of the claims involved in the appeal are as follows:

30. A pharmaceutical composition comprising a therapeutically effective amount of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells and a pharmaceutically acceptable carrier therefor,

said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine,

said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of the liposome through a maleimide group,

said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane, and

said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:5 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:6.

31. The pharmaceutical composition of claim 30, wherein the phosphatidylethanolamine is maleimidated phosphatidylethanolamine.

32. The pharmaceutical composition of claim 30, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine.

33. The pharmaceutical composition of claim 30, wherein the anti-cancer agent or toxin to cancer is selected from the group consisting of adriamycin, daunomycin, mitomycin, cisplatin, vincristine, epirubicin, methotrexate, 5Fu, aclacinomycin, ricin A and diphtheria toxin.

34. The pharmaceutical composition of claim 30, wherein the monoclonal antibody fragment is a $F(ab')_2$ antibody fragment.

35. A pharmaceutical composition comprising a therapeutically effective amount of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells and a pharmaceutically acceptable carrier therefor,

said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine,

said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of the liposome through a maleimide group,

said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane,

said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:11 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:12.

36. The pharmaceutical composition of claim 35, wherein the phosphatidylethanolamine is maleimidated phosphatidylethanolamine.

37. The pharmaceutical composition of claim 35, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine.

38. The pharmaceutical composition of claim 35, wherein the anti-cancer agent or toxin to cancer is selected from the group consisting of adriamycin, daunomycin, mitomycin, cisplatin, vincristine, epirubicin, methotrexate, 5Fu, aclacinomycin, ricin A and diphtheria toxin.

39. The pharmaceutical composition of claim 35, wherein the monoclonal antibody fragment is a $F(ab')_2$ antibody fragment.

40. A liposome/antibody conjugate consisting essentially of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells,

said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine,

said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of a liposome through a maleimide group,

said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane, and

said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:5 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:6.

41. The liposome/antibody conjugate of claim 40, wherein the phosphatidylethanolamine is maleimidated phosphatidylethanolamine.

42. The liposome/antibody conjugate of claim 40, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine.

43. The liposome/antibody conjugate of claim 40, wherein the anti-cancer agent or toxin to cancer is selected from the group consisting of adriamycin, daunomycin, mitomycin, cisplatin, vincristine, epirubicin, methotrexate, 5Fu, aclacinomycin, ricin A and diphtheria toxin.

44. The liposome/antibody conjugate of claim 40, wherein the monoclonal antibody fragment is a $F(ab')_2$ antibody fragment.

45. A liposome/antibody conjugate consisting essentially of a monoclonal antibody fragment bound to the surface of a liposome enclosing an anti-cancer agent or toxin to cancer cells,

said liposome comprising phosphatidylcholine, cholesterol and phosphatidylethanolamine,

said liposome being modified with poly(ethylene glycol), wherein the poly(ethylene glycol) is bound to the surface of a liposome through a maleimide group,

said antibody fragment belonging to IgG class or IgM class and specifically binding to a surface antigen of a stomach and colon cancer cell membrane, and

said antibody fragment having a variable region of the heavy chain which comprises the amino acid sequence shown in SEQ ID No:11 and having a variable region of the light chain which comprises the amino acid sequence shown in SEQ ID No:12.

46. The liposome/antibody conjugate of claim 45, wherein the phosphatidylethanolamine is maleimidated phosphatidylethanolamine.

47. The liposome/antibody conjugate of claim 45, wherein the phosphatidylcholine is dipalmitoylphosphatidylcholine.

48. The liposome/antibody conjugate of claim 45, wherein the anti-cancer agent or toxin to cancer is selected from the group consisting of adriamycin, daunomycin, mitomycin, cisplatin, vincristine, epirubicin, methotrexate, 5Fu, aclacinomycin, ricin A and diphtheria toxin.

49. The liposome/antibody conjugate of claim 45, wherein the monoclonal antibody fragment is a $F(ab')_2$ antibody fragment.

X. OTHER MATERIALS THAT APPLICANTS CONSIDER NECESSARY.

Enclosed herewith for the Board's convenience are copies of the Rule 1.132 Declarations and references noted in the arguments above.

Respectfully submitted,

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Antibody-directed liposomes as drug-delivery vehicles

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Key words: Liposome; Drug targeting; Antibody-liposome conjugation; Liposome–cell interaction; Liposome targeting

Contents

Abbreviations: RES, reticuloendothelial system; SUV, small unilamellar vesicle; PE, phosphatidylethanolamine; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; SPDP, *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate; DTP, dithiopyridine; MPB, *N*-[4-(*p*-maleimidophenyl)butyryl]; LUV, large unilamellar vesicle; REV, reverse-phase evaporation vesicle; SATA, succinimidyl-S-acetylthioacetate; SAMSA, S-acetylmercaptosuccinic anhydride; AETA, aminoethylthioacetyl; NHSP, *N*-hydroxysuccinimide ester of palmitic acid; FITC, fluorescein isothiocyanate; MLV, multilamellar vesicle; MTX, methotrexate; DNP-cap, *N*-dinitrophenylamino caproyl; CF, carboxyfluorescein; DOPC, dioleoylphosphatidylcholine; TNP, trinitrophenyl moiety; PHC, palmitoyl homocysteine; CHEMS, cholesteryl hemisuccinate; DOPE, dioleoylphosphatidylethanolamine; RET, resonance energy transfer; OA, oleic acid; ANTS, 1-aminonaphthalane-3,6,8-trisulphonic acid; DPX, *N,N'-p*-xylylenebis (pyridinium bromide); NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; ADP, adenosine diphosphate; HSV, herpes simplex virus; TK, thymidine kinase; PEP, phospho*enol*pyruvate; CK, carboxykinase; CAT, chloramphenicol acetyltransferase; AFP, anti-human α-fetoprotein; MHC, major histocompatibility complex; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; TPE, phosphatidylethanolamine transphosphatidylated from egg phosphatidylcholine

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Summary

The field of liposome targeting has developed rapidly in the past 10 years as a multidisciplinary approach to the controlled delivery of bioactive molecules. Liposomes as delivery vehicles offer several benefits over the administration of free compounds as therapeutic devices. Advantages such as the ability to encapsulate a wide variety of polar, nonpolar, and amphipathic agents make the liposome an attractive delivery device for many applications. Encapsulation protects the drug from metabolic degradation and protects host tissue from non-specific effects until their arrival at the site of consumption. The large relative carrying capacity of the liposomes permits the transfer of thousands of drug molecules to a given cell. Regardless of the type of liposome or its route of administration, the primary consumer of either empty or drug-laden liposomes are the highly phagocytic members of the reticuloendothelial system. Thus, except for these 'natural' target cells, liposomes remain relatively non-specific transporters. Conjugation of targeting ligands such as antibodies to the drug or drug carrier confers specificity of that complex for a certain cell or organ expressing the targeted antigenic determinant. However, direct conjugation of drug with antibody has met with limited success, due in part to the required chemical coupling between the drug and antibody. Alternatively, by first entrapping a native drug into a liposome by a non-perturbing method, followed by conjugation of antibodies to the surface of the liposome, some of the advantages of either independent approach may be realized without many of the disadvantages. The pros and cons of this general approach have been debated during the past decade in many comprehensive reviews and monographs that have been published on the subject of liposome targeting. The primary emphasis of the current effort is to focus on one particular strategy for liposome targeting, i.e., the use of antibodies as vector molecules for targeted liposome drug delivery systems. In the sections that follow we try to describe the progress realized and the pitfalls and the limitations encountered with this approach to drug targeting.

age of vesicle contents reported. Thiolated IgG exhibits very little non-specific binding to SUV's [34]. This method is attractive in its simplicity and the adsorptively coated liposomes exhibit considerable stability even in the presence of serum [27,28,30]. However, the mode of attachment is non-specific and the coating efficiency is highly variable. Even though Huang and Kennel [28] found no large interspecies differences in the adherent capacity of normal IgG, there may be differences in IgG subtype for a given liposome [30].

There are two major approaches which have been developed for the specific and controlled coating of liposomes with antibody. The first approach is to derivatize lipid, typically the amino group of phosphatidylethanolamine (PE), which, following incorporation into the liposome, will covalently attach antibody which is subsequently applied. The second major approach is to convert an otherwise hydrophilic protein antibody molecule into an amphipathic molecule which can then non-covalently intercalate into the liposomal bilayer. We will discuss briefly only some of the more popular techniques along with their most recent modifications. Examples of these procedures are listed in Table I.

II.1. Covalent conjugation to pre-formed liposomes

One of the first successful attempts at covalent attachment of antibody to liposomes was achieved by glutaraldehyde conjugation. The traditional use of glutaraldehyde has been for cell fixation in which proteins are effectively cross-linked via their amino groups. Under the proper conditions, glutaraldehyde can also be used for controlled conjugation of amino groups between antibody and PE. Following their earlier success with liposome immobilized α-chymotrypsin [35], Torchilin and co-workers [36] used glutaraldehyde to conjugate polyclonal Ig to PE-containing SUV's. These immunoliposomes retained antigen binding capacity both in vitro and in vivo and although up to 60% coupling efficiency was achieved, the net antibody incorporation was low. Potential undesirable side reactions would be homocoupling between proteins or between liposomes which would limit its general usefulness.

Another fundamental conjugation method involves coupling of protein carboxylic groups with the primary amine of PE on the liposome. Coupling between antibody and liposome has been achieved with a water soluble carbodiimide (EDCI) [37]. A major drawback is potential protein–protein cross-linking but protection of the protein amines by prior citraconylation minimizes this side reaction [37,38]. Further, this method is inefficient and produces unstable liposomes which retain little of entrapped contents [37,38]. More recently, Kung and Redemann [39] have used carboxyacyl derivatives of PE for conjugation to protein amines using EDCI. PE was derivatized with C4 to C18 dicarboxylic acids which provide spacer arms of various chain lengths. Maximum antibody incorporation was found for 1,12-dodecanedicarboxylic acid (C16). Incorporation was dependent upon the spacer length and the shortest derivative, succinic (C6), produced no detectable protein binding.

Protein attachment to liposomes can also be achieved via simple Schiff base formation under the proper conditions [40–42]. Oxidation by periodate of the car-

bohydrate moieties on glycolipid-containing liposomes to the corresponding aldehydes facilitates Schiff base formation with protein amines. The conjugate is then stabilized by subsequent reduction with sodium borohydride or sodium cyanoborohydride. Coupling efficiencies of up to 200 μg IgG per μmol lipid has been achieved [40,41]. These liposomes showed greater binding capacity to target cells than immunoliposomes produced by earlier conjugation techniques. Potential drawbacks of this technique are that 9–20 mol% of glycolipid must be included in the liposome and that periodate addition to the pre-formed liposomes may oxidize the fatty-acyl chains of the lipid and/or the contents within the liposome. In addition, up to a 60% reduction of immunoreactivity of antibodies following periodate oxidation has been observed [42]. Aldehyde groups can alternatively be generated from the carbohydrate groups on the constant region of the heavy chain of immunoglobulins by mild oxidation with periodate or glucose oxidase [43]. Addition of oxidized antibody to liposomes containing a hydrazide group resulted in up to 535 μg of IgMs per μmol phospholipid [43].

One of the most popular conjugation methods utilizes the heterobifunctional agent *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP) [44]. In this scheme, SPDP is reacted with liposomal-PE to produce the dithiopyridine derivative, DTP-PE, which can then react by disulfide interchange with any protein having a free thiol [44–47]. Immunoglobulin Fab' fragments are thus potential coupling partners with yields up to 600 μg Fab' per μmol lipid [45]. Typically 1–5 mol% of DTP-PE is incorporated into the liposome with complete retention of entrapped molecules [44–47]. If intact antibody is used (following SPDP modification) then greater than 60% of antibody-conjugated liposomes could be precipitated by *Staphylococcus aureus* indicating exposed antibody Fc regions on the liposome surface. Conjugation of antibody Fab' via the free SH is thus disadvantageous for in vivo applications because if the Fc portion remains intact and exposed, the immunoliposomes can be trapped by Fc receptor-bearing cells present in the host. Advantages of this method are a lack of homocoupling between proteins or liposomes, provision of a spacer arm, and the stability of liposomal-PE-DTP and antibody-DTP. The primary disadvantage is that the disulfide product is susceptible to reductive cleavage by endogenous thiols and would therefore be generally unsuitable as an in vivo targeting vehicle.

Goundalkar et al. [48] demonstrated that derivatized fatty acid could also be used as an anchor for antibody conjugation to pre-formed liposomes. Stearylamine was reacted with SPDP and then incorporated into liposomes. Thiolated antibody could then be conjugated to the liposomes with a 24–32% binding efficiency. Approx. 20% of an encapsulated drug leaked out during the latter binding reaction. In 1982, Martin and Papahadjopoulos [49] introduced a new sulfhydryl-reactive lipid derivative *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE). When reacted with a thiol-bearing antibody or Fab' fragment, a stable thioether bond is formed between the two by sulfhydryl addition across the maleimide double bond. Coupling efficiency is 20–30%, producing up to 600 μg Fab' per μmol lipid corresponding to about 3000 Fab' per 0.2 μm vesicle. The advantage of this protocol over that with SPDP is that the conjugation is essentially irreversible and

the covalently linked product is stable in serum [49]. For most of the earlier targeting strategies, SUV's were most often employed. However, large unilamellar vesicles (LUV's) such as those produced by the reverse-phase evaporation technique [50] have larger internal volume and better trapping efficiencies. Reverse-phase evaporation vesicles (REV's) conjugated to antibody with this derivative are stable and do not aggregate or leak [49]. In addition, they exhibit good binding and retention of contents even in 50% serum [49]. However, depending upon the lipid composition, high concentrations of the derivative can perturb membranes. Coupling of Fab' to liposomes with 5 mol% MPB-PE caused increases in size, polydispersity and resulted in up to 95% loss of entrapped carboxyfluorescein [51]. Others have reported that up to 10 mol% MPB-PE could be used without any adverse effects on bilayer structure [49]. Again, these discrepancies are probably largely due to differences in lipid composition and methods used for preparation of liposomes.

MPB-PE or DTP-PE have been used with success for conjugation of Fab' which has a free thiol available for conjugation following cleavage of $F(ab')_2$ with dithiothreitol (DTT). If the protein has no free sulfhydryl groups available, then it can be thiolated with a variety of agents (Table II). In fact, SPDP was originally designed to introduce sulfhydryl groups into proteins for reversible protein–protein conjugation [52] and was therefore the early choice to thiolate antibodies [44,46,47]. It is necessary that soluble $F(ab')_2$ or Ig-DTP be first reduced, the products of which are unstable and must be used immediately. In 1983, Duncan et al. [53] introduced a new thiolating agent, succinimidyl-S-acetylthioacetate (SATA). This agent has the advantage that deprotection of the sulfhydryl is accomplished with hydroxylamine at neutral pH and does not necessitate removal of strong maleimide-reactive reducing agents prior to addition to derivatized liposomes. SATA has been used to conjugate up to 300 μg Ig per μmol lipid into MPB-PE sensitized liposomes [54]. S-acetylmercaptosuccinic anhydride (SAMSA) is very similar to SATA but alters the electrostatic properties of the protein. Traut's reagent (2-iminothiolane) [55] has been used to thiolate many proteins including monoclonal antibody [56] and has the advantage that no deprotection is required.

There have been several novel methods developed for the indirect coupling of

TABLE II

METHODS OF INTRODUCTION OF THIOLS INTO PROTEINS

Protein	Thiolation reagent	Activating agent	Refs.
Polyclonal (rabbit) antibodies	*N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)	dithiothreitol (DTT)	52
Monoclonal IgG	SPDP	DTT	44,46
Protein A	SPDP	DTT	44,47
γ-globulin	Succinimidyl-S-acetyl thioacetate (SATA)	hydroxylamine	53,54
IgG	S-acetylmercaptosuccinic anhydride (SAMSA)	hydroxylamine	34
Monoclonal IgG	2-Iminothiolane (Traut's reagent)	none	55,56

antibody with liposomes. These systems are designed primarily for targeting of liposomes to cells with derivatized antibody already bound to their surface. Two principal schemes have been utilized based upon the affinity between protein A of *S. aureus* and antibody Fc, and the affinity of biotin for avidin. Leserman and coworkers [44,57,58] conjugated protein A to liposomes after both had been derivatized with SPDP. Protein A-bearing liposomes are added to cells which had been pre-treated with the targeting antibody resulting in specific binding of the liposomes of the cells with the newly added Fc. A similar mechanism is employed with the biotin-avidin system [59–61]. Typically, liposomes and antibody are both derivatized with biotin. Target cells bound with biotinylated antibody are then treated with avidin followed by the biotinylated liposomes. Avidin has the capacity to bind up to four biotin molecules with very high affinity, thus serving to cross-link the two biotinylated components. Streptavidin has recently been used to avoid the nonspecific binding properties of native avidin [59].

II.2. Incorporation of lipophilic antibodies

The second major approach to incorporate antibody into liposomes is to add a hydrophobic moiety to the antibody which facilitates its association with the amphipathic bilayer. Sinha and Karush [62] were the first to attempt this approach utilizing PE and the hydrophobic anchor. An alkylating phospholipid derivative, *N*-(*N*-iodoacetyl-*N*-dansyllysyl)-PE, was used to derivatize Bence-Jones monomers which then spontaneously attached to either preformed liposomes or red cell ghosts. However, this iodoacetyl derivative of PE is not an effective crosslinking agent for coupling thiolated protein into pre-formed liposomes [34]. Addition of one or two aminoethylthioacetyl (AETA) groups as spacers between iodoacetyl and PE groups makes it a reasonably efficient surface target for conjugation with thiolated antibody [34]. Periodate oxidation of the carbohydrate unit of phosphatidylinositol to a free aldehyde permits Schiff base formation between protein and phospholipid [63]. Derivatized protein can then be incorporated into liposomes by detergent dialysis. Yet another approach utilizes a polysaccharide derivative of cholesterol as the anchoring molecule [64]. The polysaccharide used in these studies, pullulan, enhanced the stability of the immunoliposomes without affecting its target specificity.

The most commonly used nonpolar anchor is a fatty acid such as palmitic acid. Two different protocols have been developed for conjugating fatty acid onto antibody. The *N*-hydroxysuccinimide ester of palmitic acid (NHSP) is one popular choice for modifying antibody to make it more lipophilic. Huang, A. et al. [65] were the first to use this agent for incorporation of monoclonal antibody into liposomes. The antibody is first reacted with NHSP with the extent of derivatization controlled by the input ratio of NHSP to Ig and/or the reaction time [66]. Optimal coupling stoichiometry for incorporation into detergent dialysis liposomes was 4–5 palmitoyl chains per IgG [66–68]. Under these conditions, about 90% of the acyl chains were located on the heavy chain of the antibody. Antigen binding capacity of the antibody was reduced 3–4-fold but the liposomes exhibited good target cell

Chen, P. S., Toribara, T. Y., and [redacted]ner, H. (1956), *Anal. Chem. 28*, 1756.
Davis, K. A., and Hatefi, Y. (1969), *Biochemistry 8*, 3355.
Hanstein, W. G., Davis, K A., and Hatefi, Y. (1971), *Arch. Biochem. Biophys. 147*, 534.
Hatefi, Y., and Hanstein, W. G. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1129.
Hatefi, Y., and Hanstein, W. G. (1973), *Methods Enzymol.* (in press).
Huang, F., and Kalant, N. (1968), *Can. J. Biochem. 46*, 1523.
Hudson, B. G., and Spiro, R. G. (1972), *J. Biol. Chem. 247*, 4229.
Kefalides, N. A. (1972), *Connect. Tissue Res. 1*, 3.
Kefalides, N. A., and Winzler, R. J. (1966), *Biochemistry 5*, 702.
Koffler, D., Sandson, J., Carr, R., and Kunkel, H. G. (1969), *Amer. J. Pathol. 54*, 293.
Krakower, C. A., and Greenspon, S. A. (1951), *Arch. Pathol. 51*, 629
Lerner, R. A., Glas[redacted], R. J., and Dixon, F. J. (1967), *J. Exp. Med. 126*, 989.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Marquardt, H., and Wilson, C. B. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 3096.
Marquardt, H., Wilson, C. B., and Dixon, F. J. (1973), *Kidney Intern. 3*, 57.
McPhaul, J. J., and Dixon, F. J. (1970), *J. Clin. Invest. 49*, 308.
Miller, E. J. (1972), *Biochemistry 11*, 4903.
Myers, C., and Bartlett, P. (1972), *Biochim. Biophys. Acta 290*, 150.
Rothbard, S., and Watson, R. F. (1972), *Lab. Invest. 27*, 76.
Spiro, R. G. (1967), *J. Biol. Chem. 242*, 1915
Tan, E. M., and Kunkel, H. G. (1966), *J. Immunol. 96*, 464.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Wilson, C. B., and Dixon, F. J. (1973), *Kidney Intern. 3*, 74.

Methyl 4-Mercaptobutyrimidate as a Cleavable Cross-Linking Reagent and Its Application to the *Escherichia coli* 30S Ribosome†

Robert R. Traut,* Alex Bollen, Tung-Tien Sun, John W. B. Hershey, John Sundberg, and L. Ross Pierce

ABSTRACT: The compound, methyl 4-mercaptobutyrimidate, of which the synthesis is described, has been used to produce disulfide-linked dimers and higher oligomers between neighboring proteins on the intact 30S ribosome from *Escherichia coli*. The imidate function of the reagent is first allowed to react with amino groups on ribosomal proteins, following which the particles modified by the addition of extra SH groups are oxidized under mild conditions to form disulfide cross-links. The formation of new products of molecular weight greater than existing protomeric ribosomal proteins is monitored by gel electrophoresis in the presence of sodium dodecyl sulfate. Upon reduction of the oxidized ribosome or the extracted proteins, the normal protein pattern on gels in sodium dodecyl sulfate is completely restored. Similarly the reduction products, or proteins bearing extra SH groups, retain their characteristic behavior during electrophoresis in buffers containing 8 M urea at pH 8.6 and 4.5. Identification of the specific protein components of purified dimers by two-dimensional slab gel electrophoresis is thus feasible. Possible minor alterations in electrophoretic mobility due to partial ionization of SH groups are completely avoided by alkylation of the proteins with iodoacetamide. The compound thus represents a new cleavable cross-linking reagent which should be applicable not only to the investigation of the topography of ribosomal proteins but also protein–protein interactions in a variety of other biological systems.

Of major importance in current research on the mechanism of protein synthesis and ribosome structure is the determination of the spatial arrangement of ribosomal proteins and the identification of ribosomal binding sites for initiation and elongation factors. Recent work has concentrated on the protein topography of the *Escherichia coli* 30S ribosomal subunit. A variety of methods are being used toward these ends (for a complete review, see Wittmann and Stoffler, 1972).

One of the most direct experimental approaches for the determination of the overall topography of ribosomal proteins as well as the identification of those specific proteins comprising the binding sites for protein factors involved in protein synthesis is the use of protein-specific bifunctional reagents to cross-link either neighboring ribosomal proteins or ribosome-bound factors and ribosomal proteins. At the time bisimido esters (Bickle *et al.*, 1972; Clegg and Hayes, 1972; Lutter *et al.*, 1972) and bismaleimides (Chang and Flasks, 1972; Kurland *et al.*, 1972) have been used to identify certain pairs of 30S ribosomal proteins covalently joined by the reagent employed.

Several approaches are available for the identification of

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the components of purified cross-linked products: (1) cleavage of the dimer or oligomer into its constituent protomers; (2) selective labeling of individual proteins in the ribosome by reconstitution techniques and demonstration of the label in specific cross-linked products; (3) analysis of purified cross-linked products with antibodies specific for each pure 30S protein. The first approach would seem to be the simplest provided that cleavable bifunctional reagents which would not alter the electrophoretic and chromatographic properties of the resulting protomers were available. Early experiments of this kind in this laboratory (Bickle *et al.*, 1972) employed bisimido esters (Davies and Stark, 1970), which seemed highly suitable for studies of this kind, first, because ribosomal proteins are rich in potentially reactive lysine amino groups; second, because the covalent bond formed is in principle cleavable by ammonolysis. Although a number of specific cross-linked products were formed, as indicated by analysis by electrophoresis on sodium dodecyl sulfate gels, identification of their protomeric components by ammonolysis was hindered by the low yield of the cleavage reaction. Therefore, we have sought new bisimido esters which could be cleaved more efficiently than the simple bisimido esters used in our earlier studies. The report by Perham and Thomas (1971) of the synthesis of a mercaptopropionimidate[1] which can introduce additional thiol groups to proteins suggested the application of this class of reagents to protein–protein cross-linking. Oxidation of the SH-charged ribosome should produce disulfide bonds between neighboring ribosomal proteins and the dimers or oligomers thus formed should be readily cleaved by mild reduction.

The present report describes improved methods for the synthesis of mercaptopropionimidate and a mercaptobutyrimidate in high yield and preliminary results on the application of these reagents to the cross-linking of 30S *Escherichia coli* ribosomal proteins. The typical procedure consists of three steps: (1) reaction of amino groups on ribosomal proteins in the intact ribosome with the imidate function of the mercaptoimidate; (2) mild oxidation of the SH-charged ribosome to form S–S linked protein dimers (or higher oligomers). The cross-linked products thus formed are readily observed on sodium dodecyl sulfate gels and can be isolated by conventional methods. (3) Reductive cleavage of the cross-linked products results in essentially 100% yield to the original protein monomers. The resulting monomeric proteins, although modified by conversion of the original lysine amino groups to mercaptobutyroamidine groups, retain their normal electrophoretic mobilities in several different gel electrophoresis systems when the SH-charged proteins are first treated with iodoacetamide. The identification of the cross-linked monomers by the conventional methods used for analysis of ribosomal proteins can therefore be readily accomplished. The method should be generally applicable to studies of protein–protein interaction in a variety of other biological structures.

Methods

Organic Syntheses

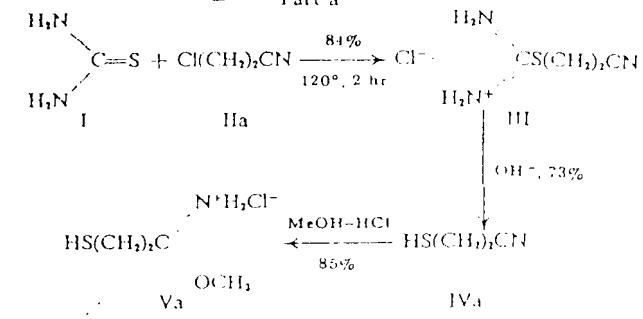
Methyl 3-Mercaptopropionimidate Hydrochloride. An improved procedure based on that of Perham and Thomas (1971) was used for the synthesis of methyl 3-mercaptopropionimidate hydrochloride. Since the overall yield (44%) is substantially greater than that previously reported, a detailed procedure is presented here. The synthesis together with yields obtained at each step is summarized in Scheme Ia. Redistilled 3-chloropropionitrile (I) (16.0 g, 0.185 mol (bp 62–63°) (11 mm)) was combined with 19.0 g (0.25 mol) thiourea (IIa) and 12.6 ml of water and refluxed in a 120° oil bath for 2 hr. After cooling the solution to room temperature, 250 ml of cold acetone was added. The crystalline isothiouronium salt, 3-isothioureidopropionitrile (III), precipitated immediately and was collected by suction filtration after standing for 15 min at 4° and washed with 3 × 50 ml of cold acetone and 2 × 50 ml of diethyl ether, and air-dried overnight (yield 25.8 g, 84%, mp 163–165°).

In the next step, 23.0 g (0.139 mol) of the isothiouronium salt (III) was dissolved in 30.0 ml of degassed H_2O and treated with 14.0 ml of 50% (w/v) NaOH solution (0.175 mol) in a 50° water bath for 30 min with stirring under nitrogen. The mixture was cooled quickly to room temperature in an ice bath, 41 ml of diethyl ether was added through an addition funnel, and the mixture was stirred for 5 min at room temperature. The reaction mixture was transferred to a nitrogen-purged separatory funnel, the ether layer was removed, and a second extraction with 41 ml of ether was performed under nitrogen. The aqueous layer was brought to pH 7 by the addition of approximately 22 ml of 6 N H_2SO_4 and extracted with 3 × 41 ml of diethyl ether under N_2. The combined ether extracts were dried 40 min with 15 g of anhydrous sodium sulfate and the solvent was removed by rotary evaporation at room temperature. The residue, 3-mercaptopropionitrile (IVa, yield 8.9 g, 73%), was obtained as a light yellow oil. This was vacuum distilled (bp 57–59° (6 mm)) and a clear colorless oil was collected.

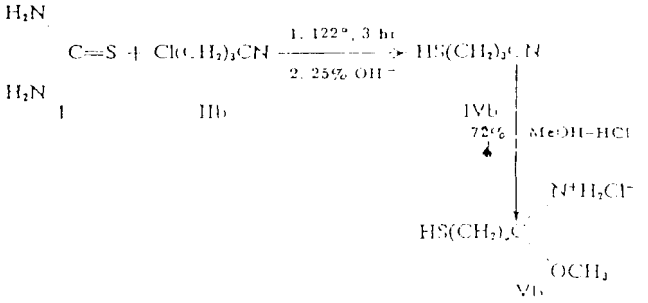
An ice-cold solution of 3.2 g (0.088 mol) of anhydrous HCl in 40 ml (0.1 mol) of absolute methanol was prepared by passing hydrogen chloride through a drying tube filled with Drierite, through concentrated H_2SO_4 in a washing bottle, and finally into the cold methanol in a reaction flask fitted with a Drierite exit tube. This was continued until the ap-

SCHEME I

Part a



Part b



[1] Abbreviations used are: TEA, triethanolamine; mercaptopropionimidate, methyl 3-mercaptopropionimidate hydrochloride; mercaptobutyrimidate, methyl 4-mercaptobutyrimidate hydrochloride.

propriate gain in weight due to added HCl was obtained. The distilled 3-mercaptopropionitrile (IVa; 2.75 g) was introduced quickly into the cold methanolic HCl and the flask was stoppered tightly and left overnight at 0°.

The reaction mixture was transferred to a three-neck flask fitted with a nitrogen inlet tube, an addition funnel for adding washing solvents, and an exit tube covered with a scintered-glass disk leading to a filtrate trap. Diethyl ether (5 ml) was added and slender white needles formed. An additional 20 ml of cold ether was added and the flask was left on ice for 1 hr. The supernatant was decanted through the scintered-glass filtered and the precipitate was washed two times with 5 ml of cold 1:3 absolute methanol–anhydrous ether and then two times with cold ether. The system was kept at 0° and under N_2 throughout these operations. The product, methyl 3-mercaptopropionimidate hydrochloride (Va; 4.2 g; 85% yield, 44% overall yield; mp 78–79° dec, lit. (Perham and Thomas, 1971) mp 73–74°) was obtained. The compound was stored in a vacuum desiccator over Drierite at 4°. Its purity was analyzed periodically on thin-layer chromatographic (tlc) plates (E. Merck, Cellulose F) by using 8:2 (95%) ethanol–ethyl acetate; spots were visualized by exposure to iodine vapor. The compound decomposed gradually over a period of several months.

Methyl 4-Mercaptobutyrimidate Hydrochloride. Methyl 4-mercaptobutyrimidate hydrochloride was synthesized by a procedure similar to that for methyl 3-mercaptopropionimidate (Scheme I, part b) but with minor modifications. The principal difference was that the intermediate isothiouronium salt (III) was not isolated. Redistilled 4-chlorobutyronitrile (38.3 g, 0.37 mol, bp 192–194°) was combined with 38 g of thiourea (0.5 mol) and 25.2 ml of water and the mixture was refluxed with stirring in a 122° oil bath for 3 hr. Analysis of the reaction mixture on tlc plates (Baker-Flex silica gel IBF) developed in methanol and visualized with uv or iodine vapor indicated quantitative conversion of 4-chlorobutyronitrile to a new product after 3 hr. Three-quarters of the cooled solution was then used directly for the next step in which the product was treated with 25.8 ml of 50% (w/v) NaOH (0.49 mol) under N_2 for 30 min at 40°. The oil layer which resulted was removed and the aqueous layer was extracted with 2 × 75 ml of diethyl ether, adjusted with 6 N H_2SO_4 to pH 7, and extracted with 3 × 75 ml of ether. The combined ether layers were extracted with 2 × 75 ml of H_2O and the combined water washings were reextracted with 2 × 35 ml of ether. The ether layers were combined and dried with anhydrous Na_2SO_4. The ether was removed by rotory evaporation, with the formation of 4-mercaptobutyronitrile (IV, 1.59 g, 26%) as a light yellow oil. This was purified by vacuum distillation (bp 61–64° (5 mm)). The purity of the distillate was confirmed by thin-layer chromatography (Baker-Flex silica gel IBF) using 1:1 chloroform–acetone. The imidate (V) was prepared by the addition of 1.4 g of this distillate ($HS(CH_2)_3CN$), 0.014 mol) to a solution of 1.9 g (0.052 mol) of dry HCl in 2.2 ml (0.069 mol) of absolute methanol and the mixture was held overnight at 0°. The product (1.7 g, 72%; 19% overall yield) was isolated in the manner previously described for methyl 3-mercaptopropionimidate hydrochloride. This solid product was recrystallized by refrigeration of saturated solutions of the compound in methanol–acetone mixture with the formation of large blunt white needles (mp 192–193° dec). The recrystallized product produced a single spot (R_F 0.58) on thin-layer chromatography (Baker-Flex silica gel IBF) using 8:2 (95%) ethanol–ethyl acetate plus 0.1% 2-mercaptoethanol.

Procedure for Cross-Linking Ribosomal Proteins by Disulfide Formation.

Step I. Reaction of Ribosomes with Imidate Function. Buffers containing primary or secondary amino groups are unsuitable for reactions involving imido ester, since the rate of reaction with buffer would greatly exceed that with protein amino groups. Accordingly, the buffer triethanolamine (TEA) at pH 8 was chosen as the standard condition for the reaction of mercaptoimidates with the ribosome. As shown previously (Bickle *et al.*, 1972), under appropriate ionic conditions in TEA the 30S ribosome retains its native physical properties, does not dimerize and retains substantial activity in poly(U)-directed poly(phenylalanine) synthesis. The standard buffer for the reaction was therefore TEA-HCl (50 mM, pH 8.0)–$Mg(OAc)_2$ (1 mM)–KCl (50 mM)–3% β-mercaptoethanol (TEA·SH buffer). Some experiments were carried out at 5 mM $Mg(OAc)_2$ and 100 mM KCl; the results were the same as those reported here.

The presence of a reducing agent such as β-mercaptoethanol was found advantageous to insure that the ribosomal SH groups were in the reduced state during reaction with mercaptoimidate, and that no disulfide-bond formation took place prior to the controlled second oxidation step.

The ribosomes were allowed to react with the mercaptoimidate (concentration given in text or figure legends) at 3 mg/ml in TEA·SH buffer for 20 min at 0° followed by dialysis at 5° against TEA·HCl (50 mM, pH 8.0)–$Mg(OAc)_2$ (1 mM)–KCl (50 mM) (TEA buffer) in order to remove β-mercaptoethanol and free mercaptoimidate or its hydrolysis products.

Step II. Oxidation of Ribosomes Modified by Addition of Mercaptoimidate Residues. Following dialysis, ribosomes were incubated for 30 min at room temperature in TEA buffer with the addition of H_2O_2 to give a final concentration of 40 mM.

The reaction was either stopped by adding sodium dodecyl sulfate sample buffer (without β-mercaptoethanol) and analyzed on gels immediately, or alternatively by addition of catalase to remove excess H_2O_2. In other experiments, the ribosomes were concentrated by centrifugation at 4° of the oxidizing reaction mixture and the pellets were resuspended in TEA buffer. The pattern and yield of cross-linked products was the same regardless of the procedure used. The formation of disulfide-linked products seemed to depend more on the number of SH groups introduced than the conditions used for oxidation.

Step III. Cleavage of Disulfide-Linked Proteins. The solutions of oxidized ribosomes in TEA buffer was adjusted to 3% β-mercaptoethanol (TEA·SH) and incubated for 30 min at 37°. Alternatively, the standard procedure for electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate in which the sample is heated in the presence of reducing agent was used to reduce the disulfide linkages.

30S Ribosomes. Subunits were prepared from *E. coli* strain MRE600 grown in rich medium as described previously (Eikenberry *et al.* (1970), zonal centrifugation; Fakunding and Hershey (1973), growth conditions).

Gel Electrophoresis. The following polyacrylamide gel electrophoresis systems were employed. (1) a modification of the stacking sodium dodecyl sulfate gel system first described by Laemmli (1970), modified according to R. F. Gesteland (personal communication; see legend to Figure 1 for details). (2) sodium dodecyl sulfate gel electrophoresis in gels containing 10% acrylamide in phosphate buffer as previously described (Weber and Osborn, 1969; Bickle and Traut, 1971).

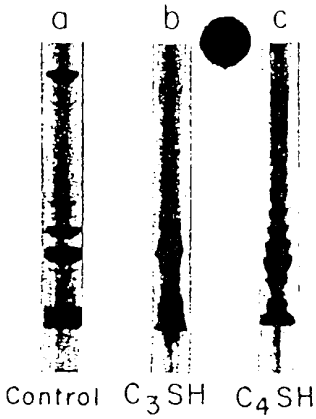


FIGURE 1: Comparison of mercaptopropionimidate and mercaptobutyrimidate. Ribosomal subunits (30 S) were treated with imidate at 10 mM, oxidized and analyzed on discontinuous polyacrylamide gels in sodium dodecyl sulfate, without mercaptan in the sample buffer. Gel system is that referred to in Methods with the following final composition: stacking gel: 5% acrylamide–2.6% bisacrylamide –0.1% sodium dodecyl sulfate–0.125 M Tris·HCl (pH 7.8); running gel: 15% acrylamide–0.08% bisacrylamide–0.1% sodium dodecyl sulfate 0.372 M Tris·HCl (pH 8.7), reservoir buffer: 0.005 M in Tris, 0.384 M in glycine, 0.1% in sodium dodecyl sulfate (pH 8.5). The sample buffer is the same as that described by Laemmli (1970): (a) control ribosomes; (b) ribosomes treated with mercaptopropionimidate; (c) ribosomes treated with mercaptobutyrimidate.

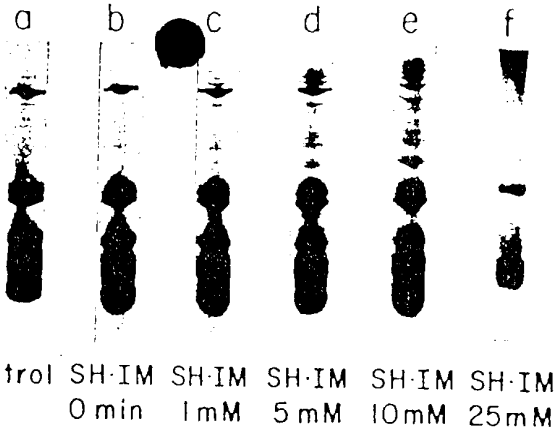


FIGURE 2: Effect of mercaptobutyrimidate concentration on the formation of products of higher molecular weight. Ribosomes were treated with mercaptobutyrimidate at the concentrations indicated and oxidized as described in Methods, and then analyzed on 10% sodium dodecyl sulfate gels in which mercaptan was omitted from the sample buffer. (d) Zero-time control at 10 mM imidate. The sodium dodecyl sulfate sample buffer was added at 0° immediately after the addition of imidate.

(3) gel electrophoresis at pH 4.5 in 8 M urea (Traut, 1966); (4) two-dimensional electrophoresis (Kaltschmidt and Wittmann, 1970; Howard and Traut, 1973). For the analysis of oxidized products reducing agents were omitted from the sodium dodecyl sulfate sample buffers, for analysis of the reduced products, reducing agents were present according to standard procedures.

Alkylation. Ribosomal proteins were extracted from control and SH-charged 30S ribosomes in TEA buffer by treatment with ribonuclease T-1 and ribonuclease A in the presence of 10 mM EDTA (Mizushima and Nomura, 1970). The precipitated proteins were solubilized by addition of solid urea to 8 M and the solution was adjusted to 17 mM iodoacetamide and allowed to react at 30° for 30 min (Battell *et al.*, 1968).

Materials

β-Mercaptoethanol was obtained from Schwarz-Mann; hydrogen peroxide AR 30% solution from Mallinckrodt Chemical Works; sodium dodecyl sulfate "Salzfrei" from SERVA, Heidelberg; 3-chloropropionitrile and 4-chlorobutyronitrile were obtained from Aldrich Chemical Co.; thiourea from Matheson, Coleman & Bell; iodoacetamide from Calbiochem. Acrylamide and bisacrylamide were used without recrystallization as obtained from Eastman Organic Chemicals. Triethanolamine was also obtained from Eastman and was redistilled before use.

Results

Comparison of Mercaptopropionimidate and Mercaptobutyrimidate. Preliminary experiments showed that both mercaptopropionimidate and mercaptobutyrimidate when allowed to react with intact *E. coli* 30S ribosomes modified the appearance of the band pattern when the proteins from the oxidized ribosomes were analyzed by sodium dodecyl sulfate gel electrophoresis. Figure 1 shows the analysis on sodium dodecyl sulfate gels of the reaction products formed with both reagents. As shown in Figure 1b, treatment of the 30S ribosome with mercaptopropionimidate resulted in the disappearance of several bands including proteins S1, S2, and S3. However, no *discrete* new cross-linked products were apparent. We suspect that the shorter compound produces mainly intramolecular cross-links, and large aggregates involving S1, S2, and S3. As shown in Figure 1c, with mercaptobutyrimidate, discrete bands of cross-linked products with high molecular weight are clearly visible. The contrast between these results comparing the two reagents led us to concentrate our efforts on the use of mercaptobutyrimidate for further studies, although it is highly likely that under appropriate conditions the shorter reagent would also prove useful for determining protein–protein interactions.

Effect of Mercaptobutyrimidate Concentration on Formation of Higher Molecular Weight Products. A variety of concentrations of mercaptobutyrimidate were tested. The results are shown in Figure 2. The standard condition 10 mM mercaptobutyrimidate was selected for further experiments in order to maximize the formation of the discrete new bands without the appearance of a preponderance of aggregates of extremely high molecular weight. The latter probably represent oligomers higher than dimers and trimers for which subsequent isolation and identification of their protomeric components would be technically complex and the results ambiguous.

Effect of Reduction Analyzed by Sodium Dodecyl Sulfate Gel Electrophoresis. As shown in Figure 2, the reaction of the ribosome with 10 mM mercaptobutyrimidate followed by oxidation resulted in the formation in discrete bands of molecular weight higher than the normal 30S gel pattern. Exposure of this cross-linked ribosome to 3% β-mercaptoethanol (see Methods) completely restores the normal sodium dodecyl sulfate gel pattern (Figure 3d). From this result, it is concluded that the formation of higher molecular weight products is caused by the formation of disulfide bridges which can be readily and completely cleaved by mild reduction with mercaptans (see Discussion).

Effect of Reduction Analyzed by One- and Two-Dimensional Urea Gel Electrophoresis. Since methods other than electro-

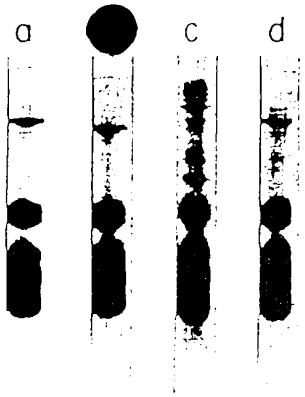


FIGURE 3: Analysis on 10% polyacrylamide gels in sodium dodecyl sulfate of ribosomes before (a) and after (b) reaction with mercaptobutyrimidate, oxidation (c), and reduction (d). The proteins were analyzed by polyacrylamide gel analysis in sodium dodecyl sulfate as referred to in Methods (Bickle and Traut, 1971). Only the sample buffer for samples b and d contain reducing agent.

phoresis in sodium dodecyl sulfate will ultimately be useful for identification of cross-linked proteins, the SH-charged ribosomal proteins extracted from ribosomes after step I were also analyzed by gel electrophoresis in buffers containing urea. After modifications of the ribosomal proteins by addition of mercaptobutyrimidate residues (40 mM imidate), changes in the mobilities of the proteins on pH 4.5 urea gels were observed both as a slight broadening or fuzziness in each band, and a systematic lowering of the mobility of all the proteins in the gel pattern (Figure 4c). The abnormal behavior could be eliminated by prior treatment of the extracted reduced protein with iodoacetamide (Figure 4d) and this was adopted as a standard procedure for analysis of the proteins in urea gel systems.

Figure 5 compares the two-dimensional gel patterns of untreated total 30S proteins with those derived from modified, oxidized, and reduced ribosomes following exhaustive treatment of the reduced proteins with iodoacetamide. Even without treatment with iodoacetamide, the patterns are similar except that the spots are less compact (results not shown). Thus, two-dimensional gel electrophoresis can be used for the identification of the components of purified cross-linked products.

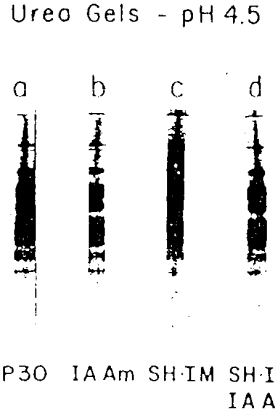


FIGURE 4: Analysis on polyacrylamide gels containing urea at pH 4.5 of 30S ribosomal proteins treated with mercaptobutyrimidate: (a) total 30S protein, untreated; (b) total protein reacted with iodoacetamide; (c) total 30S protein extracted from ribosomes following treatment with 10 mM mercaptobutyrimidate; (d) proteins from (c) after reaction with iodoacetamide.

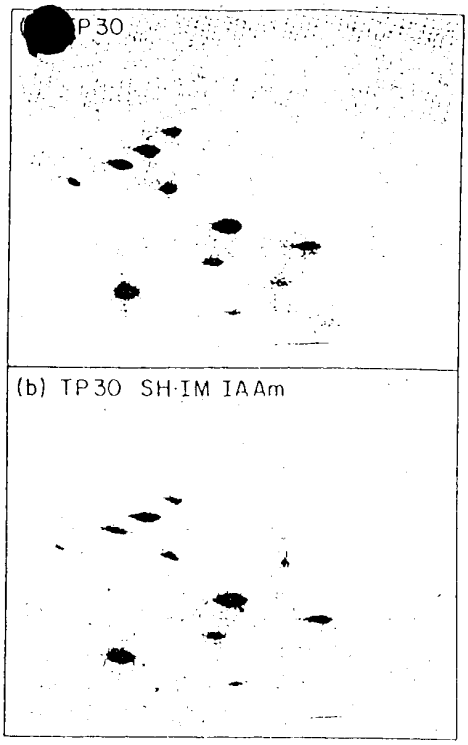


FIGURE 5: Analysis by two-dimensional polyacrylamide electrophoresis of 30S ribosomal proteins: (a) control total 30S protein; (b) protein extracted from 30S ribosomes following reaction with 10 mM mercaptobutyrimidate and carboxymethylation with iodoacetamide. Only those proteins migrating toward the cathode in the first dimension are shown. The mobility of proteins S1, S2, S6, and S10 (not shown) are also unchanged.

Analysis by Sucrose Gradient Centrifugation of Modified Oxidized, and Reduced Ribosomes. Figure 6 shows sucrose gradient analyses of 30S ribosomes at each step of the procedure adopted. The results show the following points: (1) no dimer formation between 30S particles occurs at any stage; (2) the particles sediment at 30S throughout the procedure; (3) the reaction with mercaptobutyrimidate leads to a slight broadening of the 30S peak; (4) this broadening is reversed by the oxidation step. It is concluded that the conditions used do not cause any major conformational change in the 30S ribosome and that therefore any protein–protein interactions detected by the formation of specific protein products of higher molecular weight reflect the proximity of the constituent proteins in the native 30S ribosomal particle.

Dependence of the Formation of Specific Cross-Linked Products on the Native 30S Ribosome Conformation. When total protein was first extracted from the 30S ribosome and then treated under conditions similar to those employed with the native particle (equal total protein concentration) no cross-linked material was formed (Figure 7). Therefore, it is concluded that the formation of the cross-linked products depends upon the relative orientation of the proteins involved and requires the native conformation of the 30S particle. Furthermore, this result makes it seem highly likely that random disulfide bond formation between free proteins during their extraction from the oxidized ribosome and further analysis does not occur.

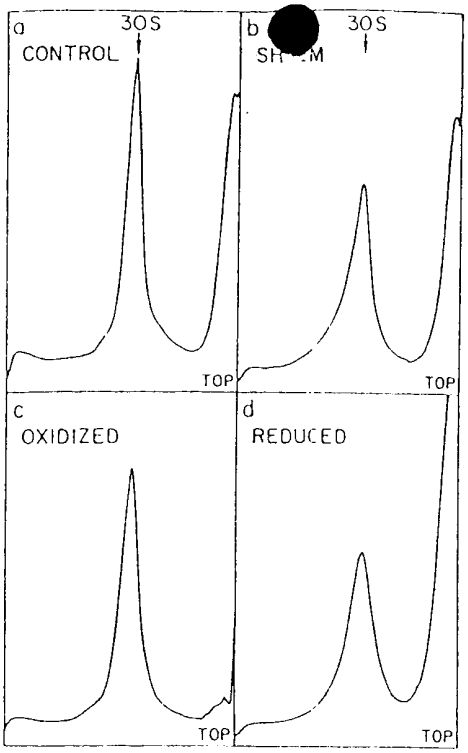


FIGURE 6: Analysis by sucrose gradient centrifugation of 30S particles at various stages of cross-linking and cleavage procedure: (a) control particles: (b) 30S ribosomes after reaction with 10 mM mercaptobutyrimidate; (c) particles from b following oxidation; (d) particles from c after reduction by incubation with 3% β-mercaptoethanol at 37° for 20 min. The ribosomes were centrifuged for 110 min at 55,000 rpm in the Beckman SW 56 rotor in linear sucrose gradients (7–25%) in TEA buffer.

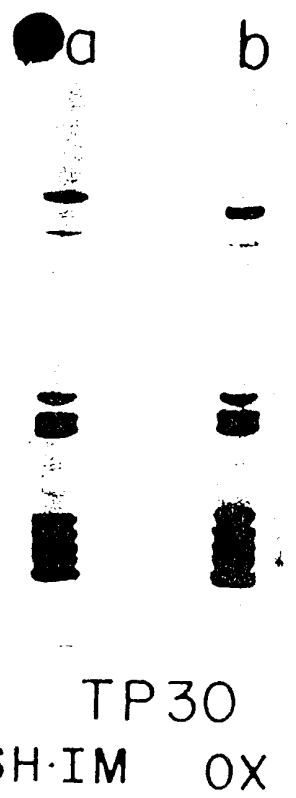


FIGURE 7: Effect of oxidation of free total 30S protein previously treated with 10 mM mercaptobutyrimidate. Total 30S protein was extracted from the ribosomal subunit as described in the text and analyzed on the discontinuous gel system described in Figure 1 in the absence of reducing agent: (a) protein from ribosomes treated with mercaptoimidate; (b) protein from (a) after the standard oxidation procedure.

Comparison of the Cross-Linking Caused by Bismethylsuberimidate and Mercaptobutyrimidate. Previous studies have shown that treatment of the 30S ribosome with bismethylsuberimidate resulted in the formation of discrete products of higher molecular weight. Figure 8 compares the spectrophotometric tracings of sodium dodecyl sulfate gels stained with Coomassie Blue of cross-linked 30S proteins prepared with bismethylsuberimidate and methyl mercaptobutyrimidate. The patterns are quite similar in the positions of the new products of elevated molecular weight; the identity of the constituents of these new products remains to be established. The experiment illustrates the greater efficiency of the mercaptobutyrimidate oxidation procedure. The concentration of this reagent was 10 mM; the concentration of bismethylsuberimidate which gave approximately the same yield of discrete cross-linked products was 25 mM, or 50 mM in total imidate. The greater efficiency of the mercaptoimidate reagent is very likely due to the fact that hydrolysis of the free imidate-terminal group of the monoreacted bisimidate competes with the cross-linking reaction to another protein. The free mercapto group, on the other hand, is stable and remains available for oxidation to the disulfide.

Discussion

A variety of methods are available and are being used in many laboratories for the formation and identification of products resulting from the treatment of ribosomal subunits with bifunctional reagents. In those cases in which the cross-linked products are not cleaved into their constituent protomeric components, rather elaborate and technically complex procedures have been employed. These studies rely either upon the availability of antibodies against each pure ribosomal protein and the testing of pure dimers one by one with each specific antibody; or on the reconstruction of ribosomes with specific proteins assumed to be cross-linked radioactively labeled. The first approach, while completely general, requires the prior preparation of purified proteins and their

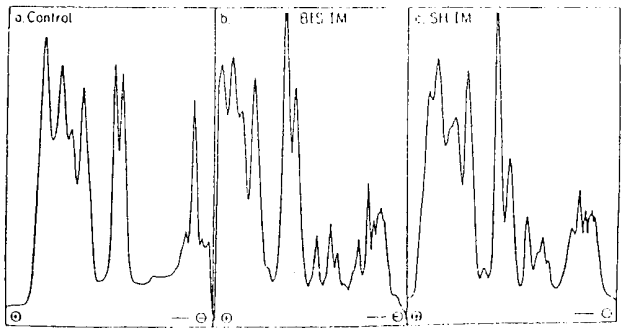


FIGURE 8: Comparison of bissuberimidate and mercaptobutyrimidate as cross-linking reagents. The 30S ribosomes were treated either with 25 mM bissuberimidate (Bickle *et al.*, 1972) or with 10 mM mercaptobutyrimidate and electrophoresed on 10% polyacrylamide gels with sodium dodecyl sulfate. The gels, following staining with Coomassie Blue, were scanned in a Gilford gel scanner (Bickle and Traut, 1971).

cognate antibodies. The s[redacted] approach is particularly applicable to confirm the composition of protein dimers when the protomeric constituents are already suggested from other results, but is not a simple general method because of the technical complexity of reconstituting ribosomes with all permutations of possible dimerizable proteins selectively labeled. A third approach might utilize an analysis of the tryptic peptides of isolated dimers or oligomers and comparison of the peptides with those already characterized from studies on pure proteins. Finally, in the exceptional case in which dimer formation proceeds in high yield, and with high specificity, the composition of a dimer may be inferred by the coordinate disappearance of putative cross-linked protomers (Chang and Flaks, 1972). Given the extremely powerful analytical techniques for identifying ribosomal proteins (*e.g.*, two-dimensional gel electrophoresis), and the need for a completely general cross-linking method which is capable of detecting even low yields of all possible cross-linked proteins without making any prior assumptions, we have concentrated our efforts on the development a readily cleavable bifunctional reagent.

The advantages of the method described here are clear. (1) Large-scale purification of ribosomal proteins is not required either to perform peptide analysis, nor to reconstruct selectively labeled ribosomes, nor to prepare antibodies. (2) The cleavage of the cross-linked products occurs in 100% yield. (3) The cleaved protomeric proteins retain their characteristic behavior in the most powerful analytical method available for ribosomal proteins, two-dimensional gel electrophoresis (Kaltschmidt and Wittmann, 1970). A modification of this technique developed in this laboratory (Howard and Traut, 1973) permits the analysis of amounts of each ribosomal protein in the range of 1–5 μg. Thus, large amounts of cross-linked materials need not be isolated; indeed, since the modified method permits radioautography, the entire procedure can be carried out on a radioactive scale. (4) All ribosomal proteins are modified by the addition of SH groups (results in progress, not shown) and thus are potentially able to form cross-links to neighboring proteins. (5) Since the mercaptoimidate is a heterogeneous bifunctional reagent, the reaction of each function can be carried out in a separate step. Thus, the method is suitable for experiments in which one protein, either a ribosomal protein or an initiation or elongation factor, is modified with the imidate function prior to its incorporation into or binding to the ribosome, after which in the controlled oxidation step, the cross-links are formed.

While it seems abundantly evident that the cross-links formed are due to disulfide-bond formation, we have compared the results of treating the SH-charged ribosomes by oxidation and by reaction with a sulfhydryl group specific bifunctional, bisphenylmaleimides (R. L. Heimark and R. R. Traut, unpublished results). The pattern of cross-linked products observed on sodium dodecyl sulfate gels was identical in two cases. This result confirms that the cross-linking is between SH groups; moreover, it indicates that the added length of possible cross-links introduced with the bismaleimide does not lead to the appearance of new products. In addition, the oxidized form of the compound, the disulfide dimer of the mercaptoimidate, has been employed directly as a bifunctional reagent. In general, the pattern of cross-linked products resembled that obtained by the procedure described here. However, yields were lower, probably due to hydrolysis of the monoreacted imido ester. Nevertheless, the disulfide diimidate should find useful application in other systems.

Preliminary experiments on the utilization of mercaptobutyrimidate[redacted]gent to identify specific *E. coli* 30S protein neighbors ha[redacted]een carried out. A number of well-separated cross-linked bands were isolated from sodium dodecyl sulfate gels with radioactive proteins. Reduction of these products resulted in their quantitative conversion to new protein bands of lower molecular weight. The molecular weights of the cross-linked bands and their reduction products as determined by sodium dodecyl sulfate gel analysis are as follows. A band with an apparent molecular weight of 35,000 gave rise on reduction to two products with molecular weights of 20,000 and 14,000. This result is entirely consistent with the dimer formation between the two monomeric proteins to form the observed dimer. Another product with an apparent molecular weight of 32,000 gave products with molecular weights of 20,000, 14,000, and 13,000, and hence is most likely a mixture of two dimers. Similarly, a third product of mol wt 26,000 yielded products of mol wt 14,000, 13,000, and 11,000 and again is likely to contain two dimers. Further identification of these products is in progress.

Other cleavable bifunctional reagents have been utilized in a variety of investigations both of the tertiary structure of single polypeptide chains and oligomeric proteins (for review, see Wold, 1972). Of these reagents two types in particular might be applicable to studies on the topography of ribosomal proteins: azo-linked bifunctional reagents cleavable in high yield by treatment with ammonia and dithionite; phenyldisulfonyl chlorides, cleavable with HBr in acetic acid. Neither class of reagent has the advantages of the reagent described here. Cleavage of the azo linkage results in a protomer in which one positive charge per amine group reacted is lost; hence, its electrophoretic mobility will be altered. The disulfonyl chlorides hydrolyze rapidly and are insoluble in water. In addition, the length of the cross-link is 5.6 Å as opposed to the 14.6-Å distance of the cross-link (N ↔ N) formed by the mercaptobutyrimidate. Previous studies with a homologous series of bis imidates differing in the number of methylene groups (T.-T. Sun, J. W. B. Hershey, R. L. Heimark, T. A. Bickle, and R. R. Traut, unpublished results) have shown that reagents shorter than bisadipimidate (8.7 Å; N ↔ N) are distinctly less effective in cross-linking ribosomal proteins.

The class of cleavable protein–protein cross-linking reagents described here should provide a valuable tool in determining the ribosomal binding sites for initiation and elongation factors and for the overall protein topography of the ribosome. They should also have a variety of applications in studies on other oligomeric enzyme complexes, more complex cellular organelles, and bacteriophage and virus structures.

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References

Battell, M. L., Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968), *J. Biol. Chem. 243*, 6206

Bickle, T. A., Hershey, J. W. B., and Traut, R. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1321.

Bickle, T. A., and Traut, R. R. (1971), *J. Biol. Chem. 246*, 6828.

Chang, F. N., and Flaks, J. G. (1972), *J. Mol. Biol. 68*, 177.

Clegg, C., and Hayes, D. (1972), *C. R. Acad. Sci. Ser. D, 275*, 1819.

Davies, G. E., and Stark, G. R. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 651.
Eikenberry, E. F., Bickle, T. A., Traut, R. R., and Price, C. A. (1970), *Eur. J. Biochem. 12*, 113.
Fakunding, J. L., and Hershey, J. W. B. (1973), *J. Biol. Chem.* (in press).
Howard, G. A., and Traut, R. R. (1973), *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett. 29*, 177.
Kaltschmidt, E., and Wittmann, H. G. (1970), *Anal. Biochem. 36*, 401.
Kurland, C. G., Green, M., Schaup, H. W., Donner, D., Lutter, L. C., and Birge, E. H. (1972), *in* Functional Units in Protein Biosynthesis, Cox, R. A., and Hadjiolov, A. H., Ed., New York, N. Y., Academic, p 75.
Laemmli, V. K. (19[illegible] *Nature* (*London*) *227*, 680.
Lutter, L. C., Zeichhardt, H., and Kurland, C. G. (1972), *Mol. Gen. Genet., 119*, 357.
Mizushima, S., and Nomura, M. (1970), *Nature* (*London*) *226*, 1214.
Perham, R. N., and Thomas, J. O. (1971), *J. Mol. Biol. 62*, 415.
Traut, R. R. (1966), *J. Mol. Biol., 21*, 571.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 224*, 4406.
Wittmann, H. G., and Stoffler, G. (1972), *in* The Mechanism of Protein Synthesis and Its Regulation, Bosch, L., Ed., Amsterdam, North Holland Publishing Co., p 258.
Wold, F. (1972), *Methods Enzymol. 25*, 623.

Rate-Determining Step in the Reconstitution of *Escherichia coli* 30S Ribosomal Subunits†

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ABSTRACT: Previous studies on the mechanism of reconstitution of 30S subunits of *Escherichia coli* using 16S RNA and unfractionated 30S proteins indicated that there is a rate-limiting, unimolecular reaction which has a high activation energy. In the present study, the 30S proteins required for this temperature-dependent, rate-limiting step have been examined. Reconstitution was done using 16S RNA and a mixture of 21 purified proteins. The proteins were divided into two parts in various ways, and reconstitution was performed in two steps. In the first step, 16S RNA was incubated with a set of proteins at a high temperature (40°) for 30 min. In the second step the remaining proteins were added and the mixture incubated at a low temperature (30°) for 10 min. It was found that the 30S ribosomal proteins S4(P4a), S8(P4b), S7(P5), S16(P9a), and S19(P13) are required in the first heat step to form active 30S particles. S15(P10b) and S17(P9b) are somewhat less essential in this respect, and S11(P7), S18(P12), S9(P8), S5(P4), and S12(P10) are still less essential but must be present during the first heat step for full functional activity. Some 30S proteins which are found in isolated ribosomal intermediate (RI) particles [S20(P14), S13(P10a), and S6(P3b,c)] do not appear to be required. Some proteins which are required for the temperature-dependent step [S5(P4), S12(P10), and possibly S19(P13)] appear to bind very weakly to RI particles at low temperatures, the isolated RI particles are deficient in these proteins. The temperature-dependent step probably involves a substantial conformational change of the RI particles, as indicated by an increase in the sedimentation coefficient of the particles after heating.

Ribosomal subunits (30S) from *Escherichia coli* can be reconstituted from their dissociated molecular components (Traub and Nomura, 1968). *In vitro* reconstitution, although differing in some details from *in vivo* assembly, may provide information relevant to the latter.

Previous studies on the mechanism of the reconstitution of 30S subunits were done using 16S RNA and a mixture of unfractionated total 30S ribosomal proteins (TP30)[1] (Traub and Nomura, 1969a; Traub and Nomura, 1969b). Kinetic studies on the assembly of 30S subunits showed that the rate-limiting unimolecular reaction has a high activation energy (Traub and Nomura, 1969a). When the components of 30S subunits (16S RNA and TP30) are mixed together under the standard reconstitution conditions and incubated at 0° instead of at the higher temperature (usually 40°) required for formation of active subunits, particles are produced which sediment at about 21–22 S in low Mg^{2+} buffer, are deficient in several proteins, and have no functional activity. These particles (called "21S particles" in this paper) are activated when heated at 40° to form particles which are capable of binding the missing proteins at 0° to form functionally active 30S particles (Traub and Nomura, 1969a). The temperature dependence of this activation step suggests that the isolated 21S particles are similar to or identical with the intermediate which undergoes the rate-limiting unimolecular reaction. The following reaction scheme has been proposed.

$$\text{16S RNA} \xrightarrow{+\text{ RI proteins}} \text{RI particles} \xrightarrow{\text{heating}} \text{RI* particles} \xrightarrow{+\text{ S proteins}} \text{30S ribosomes} \quad (1)$$

† From the Institute for Enzyme Research and Departments of Biochemistry and Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received March 5, 1973*. This is paper No. 1634 from the Laboratory of Genetics and paper XX in the series Structure and Function of Bacterial Ribosomes. Paper XIX in this series is Higo *et al.* (1973). This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the National Institute of General Medical Sciences (GM-15422) and the National Science Foundation (GB-31086X1).

[1] Abbreviations used are: TP30, unfractionated total 30S ribosomal proteins; RI, reconstitution intermediate; RI*, activated reconstitution intermediate; S proteins, 30S ribosomal proteins which do not bind to RI, but bind to RI* during assembly of 30S ribosomal subunits.

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Antibody-mediated targeting of liposomes to erythrocytes in whole blood *

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Key words: Drug targeting; Liposome; Cell-specific antibody; $F(ab')_2$ fragment; (Erythrocyte)

$F(ab')_2$ fragments derived from anti-rat erythrocyte antibody or normal rabbit serum IgG were covalently attached to the surface of liposomes consisting of equimolar amounts of egg phosphatidylcholine and cholesterol. These liposomes were interacted with rat, monkey or mouse blood, and their binding to both red and white blood cells was determined. Results of these studies show that coupling of liposomes to anti-rat erythrocyte $F(ab')_2$ considerably enhances their binding to erythrocytes in rat blood. However, no such increase in the binding was observed with rat leukocytes or monkey and mouse erythrocytes. Besides, the interactions between the liposomes and target cells did not affect the permeability properties of the liposome bilayer. These observations indicate that liposomes coupled to cell-specific antibodies may serve as highly useful carriers for homing of drugs/enzymes to specific cells in biophase.

Introduction

Liposomes containing cell-specific ligands on their surface may serve as useful carriers for delivering drugs/enzymes to specific cells in biophase [1,2]. Several studies have been done on the interactions of such liposomes with isolated specific cells [3–7], but few attempts have been made to investigate their binding to selected cell types in vivo [8,9] or in whole blood [10]. We describe here the results of our studies on the interactions of anti-rat erythrocyte $F(ab')_2$-coupled liposomes with erythrocytes in whole blood.

Materials and Methods

Egg phosphatidylcholine (egg PC) and egg [*methyl*-^{14}C]PC (25–30 μCi/μmol) were prepared as described earlier [11]. Gangliosides were isolated from buffalo brain using the known method [12]. Cholesterol was purchased from Centron Research Laboratory, Bombay, and crystallized three times from methanol. Sodium cyanoborohydride, lactoperoxidase and pepsin were purchased from Sigma Chemical Company. Ficoll 400, Sepharose 6B and Sephadex G-50 were bought from Pharmacia Fine Chemicals. Conray 420 was procured from May and Baker. Sodium [^{125}I]iodide (carrier-free) and [^{14}C]sucrose were obtained from Bhabha Atomic Research Centre, Trombay, India.

Preparation of ^{125}I-labeled $F(ab')_2$

Anti-rat erythrocyte antibodies were raised in rabbits and isolated essentially according to Bratcher et al. [13]. $F(ab')_2$ from the antibodies were prepared by pepsin treatment as in Ref. 14. Contamination of the antibody from the corresponding $F(ab')_2$ was removed by chromatography of the mixture over a Protein A-Sepharose CL-4B column. Purity of the antibody (> 90%) and of $F(ab')_2$ (> 95%) was established by polyacryla-

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Abbreviation: PC, phosphatidylcholine.

mide gel (4–5.6%) and cellulose acetate paper electrophoreses. Immunoglobulins from the normal rabbit serum were isolated by ammonium sulfate precipitation, and converted into the corresponding $F(ab')_2$ using the above procedure. Lactoperoxidase-catalysed radioiodination of both the anti-rat erythrocyte $F(ab')_2$ and normal $F(ab')_2$ was carried out according to the known method [15].

Preparation of liposomes

Unilamellar liposomes were prepared from egg PC (20 μmol), cholesterol (20 μmol), gangliosides (4 μmol) and traces of either egg [^{14}C]PC (about 10 μCi) or [^{14}C]sucrose (about 125 μCi) in 0.8 ml borate-buffered saline (10 mM borate containing 60 mM NaCl, pH 8.4) by probe-type sonication, and fractionated by ultracentrifugation as described earlier [7]. Free [^{14}C]sucrose from the liposomized sucrose was removed by gel filtration over Sephadex G-50 [11]. The mean outer diameter of these liposomes, as determined by negative-staining electron microscopy, was about 40 nm.

Covalent coupling of liposomes to ^{125}I-labeled $F(ab')_2$

Anti-rat erythrocyte $F(ab')_2$ and normal $F(ab')_2$ were covalently attached to the liposome surface essentially according to the published method [16]. Briefly, liposomes were first oxidized (2 h, 20°C, dark) with sodium periodate and, after oxidation, the excess reagent was removed by gel filtration of the reaction mixture over Sephadex G-50 using 20 mM borate containing 120 mM NaCl (pH 8.4) as the eluting buffer. The liposome fractions were pooled together and concentrated to about 5–7 μmol lipid P/ml in an Amicon centriflo CF-25 cone. Variable amounts of these liposomes were mixed with fixed amounts (1.5–1.75 mg) of anti-rat erythrocyte $F(ab')_2$ or normal $F(ab')_2$ containing about 60 μCi (spec. act. 230–320 μCi/mg protein) of the corresponding ^{125}I-labeled protein in the above buffer. To it was added 2 M sodium cyanoborohydride (10 μl/ml). The mixture was incubated at 20°C for 14–16 h. After completing the incubation, it was chromatographed on a Sepharose 6B column (1.4 × 35 cm) using sucrose-supplemented Tris-buffered saline (10 mM containing 150 mM NaCl, 44 mM sucrose and 5 mM EDTA, pH 7.4) as the eluant. The liposomes were recovered in the void volume. The liposome fractions were pooled together and concentrated to about 5 μmol lipid P/ml. Less than 20% of the total added protein becomes covalently attached to the liposomes in these conditions. For the sake of convenience, the liposomes coupled to anti-rat erythrocyte $F(ab')_2$ and normal $F(ab')_2$ will hereafter be referred to as the experimental and control liposomes, respectively.

Liposome-cell interactions in blood

Liposomes (0.05–1.0 μmol lipid P) were incubated with heparinized rat, mouse or monkey blood (1.0 ml) at 37°C for 1 h in a shaking water-bath. The plasma was removed, and the pellet was washed several times with the sucrose-supplemented Tris-buffer. Red and white blood cells were isolated from the pellet using Ficoll-Conray gradient. The gradient was prepared by mixing Conray 420 (33% solution in water, w/v, based on the sodium iothalamate content) to a 9% solution (w/v) of Ficoll 400 in normal saline till the density of the mixture was 1.08. 1 ml of the cell suspension (50% hematocrit in the Tris buffer) was carefully layered on the top of 2 ml gradient in a glass tube (5 ml). It was centrifuged at 300 × g at 20 ± 2°C for 10 min. White blood cells were aspirated off, leaving the erythrocyte pellet at the bottom of the tube. Both types of cells were washed and separately suspended in measured volumes of the Tris buffer. The red cells, so obtained were contaminated with 0.1% polymorphonuclear leukocytes, whereas the white cells were virtual free from erythrocytes. The amounts of ^{125}I in the cell pellets were determined without disrupting the cells, but for measuring ^{14}C, the cells were lysed with Triton X-100. In case of erythrocytes, the amounts of ^{14}C measured were corrected for quenching by hemoglobin.

Stability of liposomes in blood

Stability of the control as well as experimental liposomes in blood was determined in terms of both the egg PC transfer to plasma proteins and the blood-induced leakage of [^{14}C]sucrose from the liposomes. For comparison, the stability of the egg PC/cholesterol liposomes that did not contain $F(ab')_2$ was also measured in identical conditions. Incubations of liposomes with blood were carried

out at 37°C for 1 h. The phospholipid transfer to plasma proteins was determined as described earlier [17]. The plasma-induced leakage of the entrapped [^{14}C]sucrose was ascertained by measuring the amounts of [^{14}C]sucrose released in the medium upon incubating (1 h, 37°C) the liposomes separately with blood and buffer. The liposomes from free sucrose were separated by gel permeation chromatography of the mixture over Sephadex G-50.

Results and Discussion

Liposomes were covalently attached to $F(ab')_2$ using the known method [16]. This method does not seem to affect the structural integrity of the liposomes bilayers, as we have observed no significant increase in the efflux rates of the entrapped solutes during and/or after the oxidation or protein-coupling steps. Incorporation of gangliosides in the egg PC/cholesterol liposome bilayer gave better protein-coupling yields than that obtained upon incorporating cerebrosides. Only 15–20% of the total amounts of added protein could be covalently attached to the liposome surface under the reported conditions [16].

Varying concentrations of liposomes, which were free of [^{14}C]sucrose but contained egg [^{14}C]PC in their bilayers, were incubated with rat blood, and the amounts of radioactivity that became associated with erythrocyte and leukocyte pellets were determined. Fig. 1 shows that the binding of liposomes to white blood cells is not altered upon attaching anti-rat erythrocyte $F(ab')_2$ to the liposomes surface. However, the binding of these liposomes to erythrocytes was at least 6-fold greater than that of the liposomes which had normal rabbit $F(ab')_2$ covalently attached to their surface. This demonstrates that covalent coupling of anti-rat erythrocyte $F(ab')_2$ to the liposome surface helps the liposomes in specifically recognizing the red cells in rat blood.

To ascertain whether the anti-rat erythrocyte antibody is specific only to rat erythrocytes, we measured binding of both the control and experimental liposomes to monkey or mouse erythrocytes in whole blood. Table I shows that the amounts of ^{14}C or ^{125}I associated with monkey or mouse red cells are not much affected upon replacing the normal rabbit $F(ab')_2$ in liposomes by the anti-rat erythrocyte $F(ab')_2$, suggesting that the antibody used in these experiments is highly specific to rat erythrocytes. In addition, the process of binding between the experimental liposomes and rat erythrocytes is very fast, as no significant increase in the binding was observed by increasing the incubation time from 15 min (^{14}C, about 14%; ^{125}I, about 16%) to 4 h (^{14}C, about 17%; ^{125}I, about 20%). Besides, the extent of the binding is not influenced appreciably by the lipo-

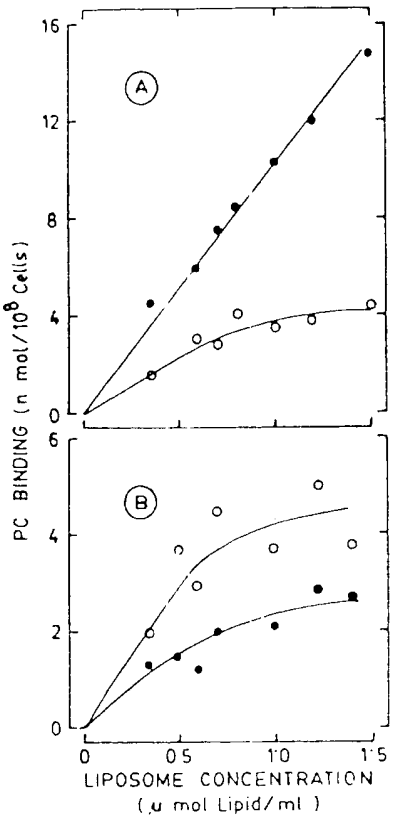

Fig. 1. Binding of liposomes to white blood cells and red cells in rat blood. The liposomes were prepared from PC, cholesterol, gangliosides and traces of egg [^{14}C]PC, and covalently attached to ^{125}I-labeled $F(ab')_2$ as given in Materials and Methods. The amounts of protein coupled to the surface of liposomes were as follows: control liposomes (B), 80–100 μg/μmol lipid P; experimental liposomes (A), 90–120 μg/μmol lipid P. The open and closed circles represent amounts of liposomal PC bound to white blood cells and red cells, respectively. For the sake of clarity, the amounts of cell-bound $F(ab')_2$ are not shown in the figure. But these amounts were quite in agreement with the amounts of cell-associated liposomal PC.

TABLE I

INTERACTIONS OF LIPOSOMES WITH MONKEY, MOUSE OR RAT ERYTHROCYTES IN WHOLE BLOOD

Liposomes (350 nmol lipid P) were incubated with monkey, mouse or rat blood (1.0 ml) at 37°C for 1 h. The amounts of $F(ab')_2$ that were covalently coupled to the surface of control and experimental liposomes were about 90 μg protein/μmol lipid P and 80 μg protein/μmol lipid P, respectively. The extents of liposome binding to erythrocytes were ascertained by measuring both the cell-associated ^{14}C and ^{125}I. Values shown are mean of three determinations ± S.D., and expressed as % of the total ^{14}C or ^{125}I associated with $3 \cdot 10^9$ red cells.

Red cells	Liposomes	^{14}C (%)	^{125}I (%)
Monkey	control	1.8 ± 1.0	0.2 ± 0.1
	experimental	2.7 ± 1.2	0.8 ± 0.3
Mouse	control	2.5 ± 0.5	0.3 ± 0.1
	experimental	1.9 ± 1.0	0.8 ± 0.2
Rat	control	3.0 ± 0.4	0.5 ± 0.1
	experimental	19.3 ± 1.0	24.0 ± 3.4

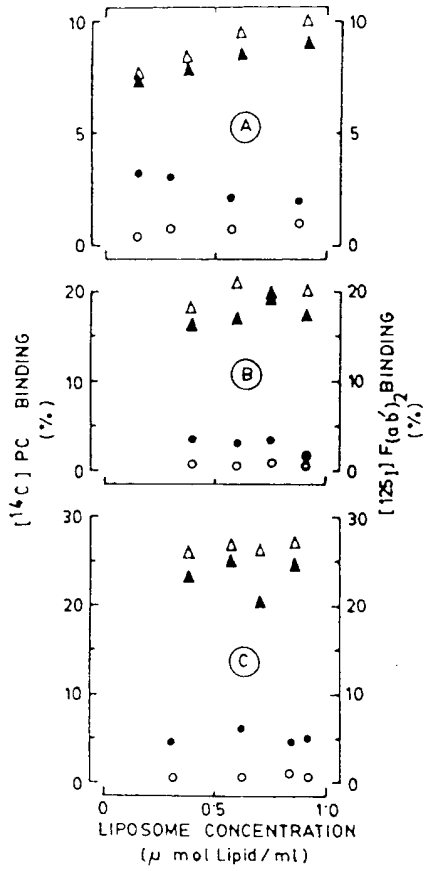

Fig. 2. Binding of liposomes to erythrocytes in whole blood. The binding is expressed as percent of total ^{14}C or ^{125}I associated with $3 \cdot 10^9$ red cells. Liposome preparations used in these experiments were similar to that in Fig. 1. The amounts of the protein coupled to the surface of control liposomes were about 12 μg/μmol lipid P (A), 76 μg/μmol lipid P (B), 1' μg/μmol lipid P (C), whereas in the case of experimental liposomes these amounts were about 8 μg/μmol lipid P (A), 70 μg/μmol lipid P (B) and 155 μg/μmol lipid P (C). The open and closed circles represent the % ^{125}I and % ^{14}C bindings, respectively, to rat erythrocytes upon incubating the control liposomes with rat blood, while in the case of experimental liposomes, the % ^{125}I and % ^{14}C bindings are represented by open and closed triangles, respectively.

some concentration in blood, but increases by increasing the protein-to-phospholipid ratio in the liposomes (Fig. 2). The latter finding is consistent with the earlier observations [3,4].

Further experiments were done to examine whether the structural integrity of the liposomes is maintained upon their binding to erythrocytes. For these experiments, [^{14}C]sucrose was entrapped in the liposomes instead of incorporating egg [^{14}C]PC in the liposome bilayer. The control and experimental liposomes were incubated with rat blood and the amounts of ^{14}C and ^{125}I present in the erythrocyte pellets were measured. Fig. 3 shows that by incubating the experimental liposomes with blood, similar amounts of ^{14}C and ^{125}I become associated with the erythrocyte pellet, suggesting that the liposomes remain largely intact even upon their binding with erythrocytes in whole blood. This was further confirmed by measuring the stability of liposomes in rat blood. The stability was measured in terms of both the phospholipid transfer to plasma proteins and the plasma-induced leakage of the entrapped [^{14}C]sucrose, as described in Materials and Methods. About 17–23% and 19–21% of egg PC was transferred to plasma proteins from the control and experimental liposomes, respectively, at 37°C in 1 h. These amounts were almost identical to those transferred from the egg PC/cholesterol liposomes (14–20%) which did not contain $F(ab')_2$ on their surface. Similarly, the extent of plasma-induced leakage of [^{14}C]sucrose from both the control (buffer, 1.4–1.8%; plasma, 2.8–3.2%) and experimental (buffer, 1.6–2.1%; plasma, 3.9–4.7%) liposomes was almost similar to

that observed for the egg PC/cholesterol liposomes (buffer, 1.1–1.6%; plasma, 1.9–2.4%). These results clearly show that liposomes consisting of equimolar amounts of egg PC and cholesterol largely remain stable in blood [18], and that this stability is not altered upon covalent coupling of anti-rat erythrocyte $F(ab')_2$ to the liposome surface.

The present study demonstrates that covalent attachment of anti-rat erythrocyte $F(ab')_2$ to the liposome surface helps the liposomes in specifically recognizing the red cells in rat blood. This antibody seems to be highly specific to these cells, as the experimental liposomes failed to bind to rat white blood cells or monkey and mouse erythrocytes. Also, the interactions between liposomes and the target cells do not appear to induce an enhancement in the leakage of the entrapped solutes in the medium, since the stability of the experimental liposomes in the presence of rat blood was found to be similar to that of the egg PC/cholesterol liposomes. Besides, the binding of liposomes to the red cells is very rapid, and not affected by the liposome concentration in blood, but it is certainly influenced by the phospholipid-to-protein ratio in the liposomes. It is, however, not yet clear from these experiments whether or not the cell-bound liposomes deliver their contents to the cells.

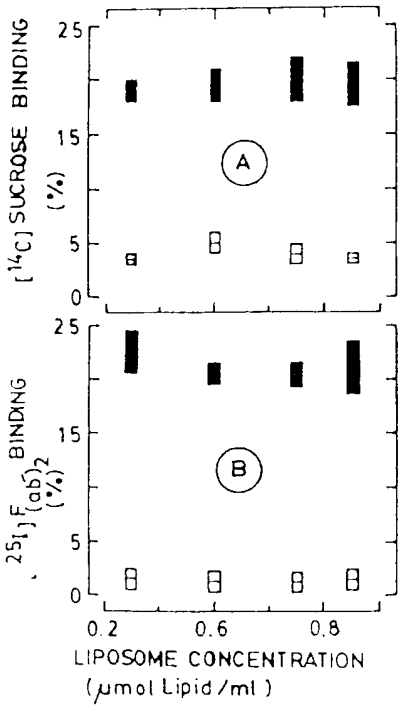

Fig. 3. Liposomes binding to erythrocytes in rat blood. The binding is expressed as percent of total ^{14}C or ^{125}I associated with $3 \cdot 10^9$ erythrocytes. ^{125}I-labeled $F(ab')_2$ were covalently coupled to the liposomes which contained [^{14}C]sucrose as the entrapped solute. The amounts of $F(ab')_2$ on the liposome surface were as follows: control liposomes, 60–75 μg/μmol lipid P; experimental liposomes, 70–90 μg/μmol lipid P. Bars represent means of three experiments ± S.D. Open bars, control liposomes; closed bars, experimental liposomes. (A) and (B) represent the % ^{14}C and % ^{125}I bindings, respectively, to erythrocytes. In one experiment, free [^{14}C]sucrose alone was incubated with rat blood for 1 h at 37°C, and the amount of radioactivity that became associated with red cells was measured. Only background levels of ^{14}C were found to be present in the erythrocyte pellet.

Delivery of liposomized solutes to the target cells will be ensured only if the cell-bound liposomes either fuse with the cell membrane or become internalized via endocytosis [19]. As a mammalian red cell should have very little or no capacity to endocytose the surface-bound liposomes, fusion between liposome bilayer and the target cell membrane seems to be the only mode of erythrocyte-liposome interaction which would result in delivery of the entrapped solutes to the cells. However, such a fusion should render the cells abnormal, and consequently might affect their survival in circulation [20]. These possibilities are presently under investigation in the authors' laboratory.

References

1 Finkelstein, M.C. and Weissmann, G. (1981) in Liposomes: From Physical Structure to Therapeutic Applications (Knight, C.G., ed.), pp. 443–464, Elsevier/North-Holland, New York
2 Gregoriadis, G. (1983) Trends Pharmacol. Sci. 4, 304–307
3 Heath, T.D., Fraley, R.T. and Papahadjopoulos, D. (1980) Science 210, 539–541
4 Harsch, M., Walther, P. and Weder, H.G. (1981) Biochem. Biophys. Res. Commun. 103, 1069–1076
5 Leserman, L.D., Machy, P. and Barbet, J. (1981) Nature (Lond.) 293, 226–228
6 Heath, T.D., Montogomery, J.A., Piper, J.R. and Papahadjopoulos, D. (1983) Proc. Natl. Acad. Sci. USA 80, 1377–1381
7 Singhal, A., Bali, A., Jain, R.K. and Gupta, C.M. (1984) FEBS Lett. 178, 109–113

8 Ghosh, P., Das, P.K. and Bachhawat, B.K. (1982) Arch. Biochem. Biophys. 213, 266–270
9 Spanjer, H.H. and Scherphof, G.L. (1983) Biochim. Biophys. Acta 734, 40–47
10 Kühn, S.H., Gemperli, B., Shephard, E.G., Joubert, J.R., Weidemann, P.A.C., Weissmann, G. and Finkelstein, M.C. (1983) Biochim. Biophys. Acta 762, 119–127
11 Gupta, C.M. and Bali, A. (1981) Biochim. Biophys. Acta 663, 506–515
12 Laine, R.A., Stellner, K. and Hakamori, S.-I. (1974) Methods Membrane Biol. 2, 214–244
13 Bratcher, R.L., Chong, C.A. and Dray, S. (1974) J. Immunol. 112, 1337–1346
14 Martin, F.J., Hubbell, W.L. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238
15 Marchalonis, J.J. (1969) Biochem. J. 113, 299–305
16 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81
17 Bali, A., Dhawan, S. and Gupta, C.M. (1983) FEBS Lett. 154, 373–377
18 Kirby, C., Clarke, J. and Gregoriadis, G. (1980) Biochem. J. 186, 591–598
19 Pagano, R.E. and Weinstein, J.N. (1978) Annu. Rev. Biophys. Bioeng. 7, 435–468
20 Rice-Evans, C.A. and Dunn, M.J. (1982) Trends Biochem. Sci. 7, 282–286